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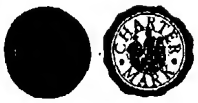

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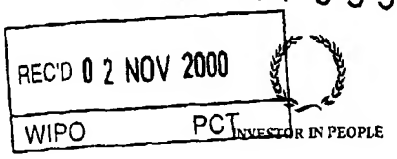

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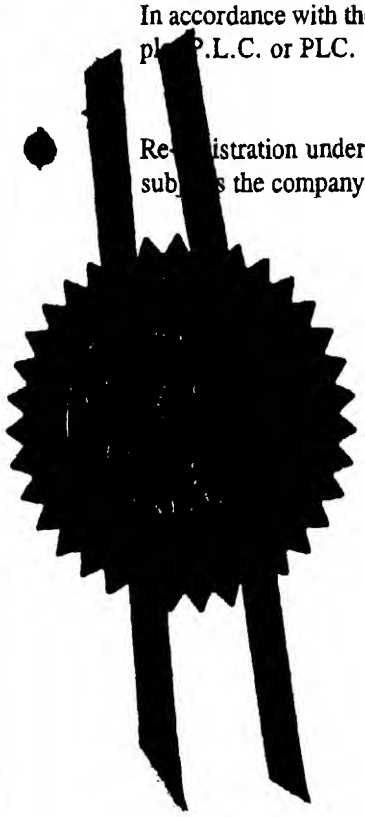

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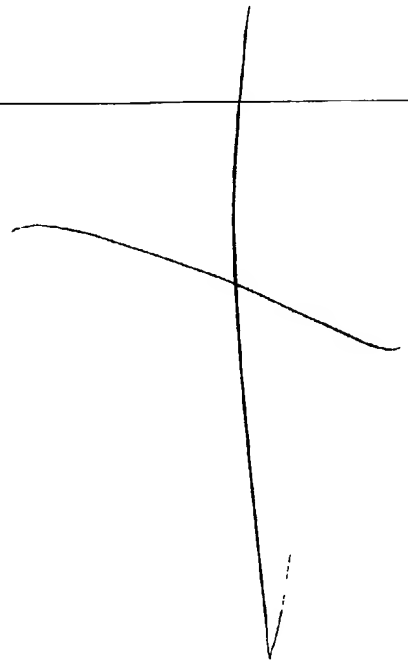

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1. Your reference — MJWD/JM/B45196

2. Patent application number
(The Patent Office will fill in his part) — **9922700.1**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — SmithKline Beecham Biologicals s.a.
rue de l'Institut 89, B-1330 Rixensart, , Belgium

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — 5781117001

4. Title of the invention — Vaccine

5. Name of your agent *(if you have one)* — CORPORATE INTELLECTUAL PROPERTY

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SMITHKLINE BEECHAM PLC
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MIDDLESEX TW8 9EP

Patents ADP number *(if you know it)* — 58009740004

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer yes if:*
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b) there is an inventor who is named as an applicant, or
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Claim(s)	2
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11. We request the grant of a patent on the basis of this application

Signature Date 24-Sep-99

M J W Dalton

12. Name and daytime telephone number of person to contact in the United Kingdom: M J W Dalton 020 8975 6348

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B45196

Vaccines

The present invention relates to a novel adjuvant system comprising a polyoxyethylene ether or ester surfactant in combination with at least one additional non-ionic surfactant. Preferably said additional non-ionic surfactant is a detergent selected from a polyoxyethylene sorbitan ester (the TWEEN™ series) and/or an Octoxynol (the TRITON™ series). The present invention provides said novel adjuvants, methods of their manufacture and their formulation into vaccines. The use of the adjuvants or vaccines of the present invention in the prophylaxis or therapy of disease is also provided.

The applicant presents here the surprising finding that polyoxyethylene ethers, in combination with at least one additional non-ionic surfactant, together act as a potent adjuvants for vaccines. Advantageously, such compositions may be administered systemically, but are sufficient to induce systemic immune responses when administered mucosally. The immune responses induced by mucosal administration of vaccines of the present invention are at least as high as those observed after a systemic injection of conventional vaccine.

Apart from bypassing the requirement for painful injections and the associated negative affect on patient compliance because of "needle fear", mucosal vaccination is attractive since it has been shown in animals that mucosal administration of antigens has a greater efficiency of inducing protective responses at mucosal surfaces, which is the route of entry of many pathogens. In addition, it has been suggested that mucosal vaccination, such as intranasal vaccination, may induce mucosal immunity not only in the nasal mucosa, but also in distant mucosal sites such as the genital mucosa (Mestecky, 1987, Journal of Clinical Immunology, 7, 265-276; McGhee and Kiyono, Infectious Agents and Disease, 1993, 2, 55-73). Despite much research in the field, safe and effective adjuvants which are suitable for use in humans, remains to be identified. The present invention provides a solution to this problem.

Medical uses of certain non-ionic surfactants have been described. For example, intranasal administration of polyoxyethylene ethers and esters for the enhancement of insulin uptake in the nasal cavity has been described (Hirai *et al.* 1981, International Journal of Pharmaceutics, 9, 165-172; Hirai *et al.* 1981, International Journal of Pharmaceutics, 9, 173-184).

Other non-ionic surfactants have been utilised in vaccine formulations. For example, vaccine preparations comprising an admixture of either polyoxyethylene castor oil or caprylic/capric acid glycerides, with polyoxyethylene sorbitan monoesters, and an antigen, are capable of inducing systemic immune responses after topical administration to a mucosal membrane (WO 94/17827). This patent application discloses the combination of the non-ionic surfactant TWEEN20™ (polyoxyethylene sorbitan monoester) and Imwitor742™ (caprylic/capric acid glycerides), or a combination of TWEEN20™ and polyoxyethylene castor oil is able to enhance the systemic immune response following intranasal immunisation. Details of the effect of this formulation on the enhancement of the immune response towards intranasally administered antigens have also been described in the literature (Gizurarson *et al.* 1996. Vaccine Research, 5, 69-75; Aggerbeck *et al.* 1997, Vaccine, 15, 307-316; Tebbey *et al.*,*Viral Immunol* 1999;12(1):41-5).

Novasomes (US 5,147,725) are paucilamenar vesicular structures comprising a non-ionic surfactant polyoxyethylene ether in combination with cholesterol to encapsulate antigen, which formulations are capable of adjuvanting the immune response to antigens after systemic administration. Non-ionic surfactants have also been formulated in such a way as to form non-ionic surfactant vesicles (commonly known as niosomes, US 5,679,355). Such vesicles, in the presence of cholesterol form lipid-bilayer vesicles which are capable of entrapping antigen within the inner aqueous phase or within the bilayer itself.

Patent application WO 96/36352 describes a liquid pharmaceutical agent comprising at least two absorption enhancers, wherein the amount of each is present in a concentration of from 1 to 10 w/w %.

Surfactants are commonly used in the formulation of oil emulsion adjuvants for systemic administration, and function to stabilise the oil droplets. For example, polyoxyethylene sorbitan esters (TWEEN™) and sorbitan fatty acid esters (SPAN™) are used to stabilise oil in water emulsions (EP 0 399 843 B, WO 95/17210).

The present invention provides safe and potent adjuvants which are easily manufactures, which may be administered either through mucosal or systemic routes. The adjuvants of the present invention comprise a polyoxyethylene ether or ester and at least one additional non-ionic surfactant.

The polyoxyethylene ethers or esters which may be formulated in the vaccines and adjuvant of the present invention comprise molecules of general formula (I):

$HO(CH_2CH_2O)_n$-A-R

wherein, n is 1-50, A is a bond or -C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

Thus, one embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, more preferably 6-12, and most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Suitable polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

Most preferably, said polyoxyethylene ether is polyoxyethylene-9-lauryl ether (laureth 9). Alternative terms or names for polyoxyethylene lauryl ether are disclosed in the CAS registry. The CAS registry number of polyoxyethylene-9 lauryl ether is: ***9002-92-0***. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12th ed: entry 7717, Merck & Co. Inc., Whitehouse Station, N.J., USA;

ISBN 0911910-12-3), where therapeutic uses are stated to include: topical anesthetic; anti-pruritic; and sclerosing agent activities. As a class, such polyoxyethylene ethers, or esters, are non-ionic surfactants. Laureth 9 is formed by reacting ethylene oxide with dodecyl alcohol, and has an average of nine ethylene oxide units.

The ratio of the length of the polyoxyethylene section to the length of the alkyl chain in the surfactant (*i.e.* the ratio of n: alkyl chain length), affects the solubility of this class of detergent in an aqueous medium. Thus, the adjuvants of the present invention may be in solution or may form particulate structures such as micelles or vesicles. As a solution, the adjuvants of the present invention safe, easily sterilisable, simple to administer, and may be manufactured in a simple fashion without the GMP and QC issues associated with the formation of uniform particulate structures. Some polyoxyethylene ethers, such as laureth 9, are capable of forming non-vesicular solutions. However, polyoxyethylene-8 palmitoyl ether ($C_{18}E_8$) is capable of forming vesicles. Accordingly, vesicles of polyoxyethylene-8 palmitoyl ether in combination with at least one additional non-ionic surfactant, to form adjuvants of the present invention.

Preferably, the polyoxyethylene ether element present in the adjuvant combinations of the present invention has haemolytic activity. The haemolytic activity of a polyoxyethylene ether may be measured *in vitro*, with reference to the following assay, and is as expressed as the highest concentration of the detergent which fails to cause lysis of the red blood cells:

1. Fresh blood from guinea pigs is washed with phosphate buffered saline (PBS) 3 times in a desk-top centrifuge. After resuspension to the original volume the blood is further diluted 10 fold in PBS.
2. 50 µl of this blood suspension is added to 800 µl of PBS containing two-fold dilutions of detergent.
3. After 8 hours the haemolysis is assessed visually or by measuring the optical density of the supernatant. The presence of a red supernatant, which absorbs light at 570 nm indicates the presence of haemolysis.

4. The results are expressed as the concentration of the first detergent dilution at which hemolysis no longer occurs.

Within the inherent experimental variability of such a biological assay, the polyoxyethylene ethers, or surfactants of general formula (I), of the present invention preferably have a haemolytic activity, of approximately between 0.5-0.0001%, more preferably between 0.05-0.0001%, even more preferably between 0.005-0.0001%, and most preferably between 0.003-0.0004%. Ideally, said polyoxyethylene ethers or esters should have a haemolytic activity similar (*i.e.* within a ten-fold difference) to that of either polyoxyethylene-9 lauryl ether or polyoxyethylene-8 stearyl ether.

To the polyoxyethylene ether or ester is added at least one additional non-ionic surfactant, which may be any detergent with suitable surface active properties. Suitable detergents are described in "Surfactant systems" Ed: Attwood and Florence (1983, Chapman and Hall). Preferred non-ionic surfactants are Octoxynols and Polyoxyethylene sorbitan esters. The Octoxynol series, including t-octylphenoxypolyethoxyethanol (TRITON X100™) is described in Merck Index Entry 6858 (Page 1162, 12th Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). The polyoxyethylene sorbitan esters, including polyoxyethylene sorbitan monooleate (TWEEN80™) are described in Merck Index Entry 7742 (Page 1308, 12th Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). Both may be purchased from Sigma Inc.

Preferably, the adjuvants of the present invention comprise a polyoxyethylene ether and a polyoxyethylene sorbitan ester. Optionally said combination may further comprise an octoxynol, such as TRITONX100™. Most preferably, said polyoxyethylene ether is polyoxyethylene-9-lauryl ether, and said polyoxyethylene sorbitan ester is polyoxyethylene sorbitan monooleate (TWEEN80™).

The prefered ranges for the concentrations of these non-ionic surfactants are:
Tween80™: 0.01 to 1%, most preferably 0.1% (v/v)
Triton X-100™: 0.001 to 0.1, most preferably 0.005 to 0.02 % (w/v).

The vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to, or suffering from disease, by means of administering said vaccine via a mucosal route, such as the oral/bucal/intestinal/vaginal/rectal or nasal route. Such administration may be in a droplet, spray, or dry powdered form. Nebulised or aerosolised vaccine formulations also form part of this invention. Enteric formulations such as gastro resistant capsules and granules for oral administration, suppositories for rectal or vaginal administration also form part of this invention. The present invention may also be used to enhance the immunogenicity of antigens applied to the skin (transdermal or transcutaneous delivery). In addition, the adjuvants of the present invention may be parentally delivered, for example intramuscular, or subcutaneous administration. When used for intranasal vaccination, the vaccines of the present invention are preferably haemolytic in nature.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from a wide variety of sources. For example, antigens may include human, bacterial, or viral nucleic acid, pathogen derived antigen or antigenic preparations, tumour derived antigen or antigenic preparations, host-derived antigens, including GnRH and IgE peptides, recombinantly produced protein or peptides, and chimeric fusion proteins.

Preferably the vaccine formulations of the present invention contain an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives

thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, ..), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (whole live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof), or derived from bacterial pathogens such as *Neisseria spp,* including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella* catarrhalis (for example high and low molecular weight adhesins and invasins*); Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof*); Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof*), C. difficile* (for example clostridium toxins A

or B and derivatives thereof); *Bacillus spp.*, including *B. anthracis* (for example botulinum toxin and derivatives thereof); *Corynebacterium spp.*, including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.*, including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp.* including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci; Leptospira spp.*, including *L. interrogans; Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola, T. hyodysenteriae;* or derived from parasites such as *Plasmodium spp.*, including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni,* or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans.*

Preferred bacterial vaccines comprise antigens derived from *Streptococcus spp,* including *S. pneumoniae* (for example capsular polysaccharides and conjugates thereof, PsaA, PspA, streptolysin, choline-binding proteins)and the protein antigen Pneumolysin (Biochem Biophys Acta, 1989, 67, 1007; Rubins et al., Microbial Pathogenesis, 25, 337-342), and mutant detoxified derivatives thereof (WO 90/06951; WO 99/03884). Other preferred bacterial vaccines comprise antigens derived from *Haemophilus spp., including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae,* for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464) or multiple copy varients or fusion proteins thereof. Other preferred bacterial vaccines comprise antigens derived from *Morexella Catarrhalis* (including outer membrane vesicles thereof, and OMP106 (WO97/41731)) and from

Neisseria mengitidis B (including outer membrane vesicles thereof, and NspA (WO 96/29412).

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS1, PreS2 S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474. In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD2t as hereinabove defined.

In a preferred embodiment of the present invention vaccines containing the claimed adjuvant comprise antigen derived from the Human Papilloma Virus (HPV) considered to be responsible for genital warts, (HPV 6 or HPV 11 and others), and the HPV viruses responsible for cervical cancer (HPV16, HPV18 and others).

Particularly preferred forms of genital wart prophylactic, or therapeutic, vaccine comprise L1 particles or capsomers, and fusion proteins comprising one or more antigens selected from the HPV 6 and HPV 11 proteins E6, E7, L1, and L2.

The most preferred forms of fusion protein are: L2E7 as disclosed in WO 96/26277, and proteinD(1/3)-E7 disclosed in GB 9717953.5 (PCT/EP98/05285).

A preferred HPV cervical infection or cancer, prophylaxis or therapeutic vaccine, composition may comprise HPV 16 or 18 antigens. For example, L1 or L2 antigen monomers, or L1 or L2 antigens presented together as a virus like particle (VLP) or the L1 alone protein presented alone in a VLP or capsomer structure. Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, and WO93/02184.

Additional early proteins may be included alone or as fusion proteins such as preferably E7, E2 or E5 for example; particularly preferred embodiments of this includes a VLP comprising L1E7 fusion proteins (WO 96/11272).

Particularly preferred HPV 16 antigens comprise the early proteins E6 or E7 in fusion with a protein D carrier to form Protein D - E6 or E7 fusions from HPV 16, or combinations thereof; or combinations of E6 or E7 with L2 (WO 96/26277).

Alternatively the HPV 16 or 18 early proteins E6 and E7, may be presented in a single molecule, preferably a Protein D- E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D - E6 or Protein D - E7 fusion protein or Protein D E6/E7 fusion protein.

The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 6, 11, 31, 33, or 45.

Vaccines of the present invention further comprise antigens derived from parasites that cause Malaria. For example, preferred antigens from *Plasmodia falciparum* include RTS,S and TRAP. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P.falciparum* linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in the International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S. TRAP antigens are described in the International Patent Application No. PCT/GB89/00895, published under WO 90/01496. A preferred embodiment of the present invention is a Malaria vaccine wherein the antigenic preparation comprises a combination of the RTS,S and TRAP antigens. Other plasmodia antigens that are likely candidates to be components of a multistage Malaria vaccine are *P. faciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in Plasmodium spp.

The formulations may also contain an anti-tumour antigen and be useful for the immunotherapeutic treatment cancers. For example, the adjuvant formulation finds utility with tumour rejection antigens such as those for prostrate, breast, colorectal, lung, pancreatic, renal or melanoma cancers. Exemplary antigens include MAGE 1 and MAGE 3 or other MAGE antigens for the treatment of melanoma, PRAME, BAGE or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al. (1997), Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other Tumor-Specific antigens are suitable for use with adjuvant of the present invention and include, but are not restricted to Prostate specific antigen (PSA) or Her-2/neu, KSA (GA733), MUC-1 and carcinoembryonic antigen (CEA). Accordingly in one aspect of the present invention there is provided a vaccine comprising an adjuvant composition according to the invention and a tumour rejection antigen.

Additionally said antigen may be a self peptide hormone such as whole length Gonadotrophin hormone releasing hormone (GnRH, WO 95/20600), a short 10 amino acid long peptide, in the treatment of many cancers, or in immunocastration.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from *Borrelia sp.*. For example, antigens may include nucleic acid, pathogen derived antigen or antigenic preparations, recombinantly produced protein or peptides, and chimeric fusion proteins. In particular the antigen is OspA. The OspA may be a full mature protein in a lipidated form virtue of the host cell (E.Coli) termed (Lipo-OspA) or a non-lipidated derivative. Such non-lipidated derivatives include the non-lipidated NS1-OspA fusion protein which has the first 81 N-terminal amino acids of the non-structural protein (NS1) of the influenza virus, and the complete OspA protein, and another, MDP-OspA is a non-lipidated form of OspA carrying 3 additional N-terminal amino acids.

Vaccines of the present invention may be used for the prophylaxis or therapy of allergy. Such vaccines would comprise allergen specific (for example Der p1) and allergen non-specific antigens (for example peptides derived from human IgE, including but not restricted to the stanworth decapeptide (EP 0 477 231 B1)).

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-500 μg, preferably 1-100μg, most preferably 1 to 50μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The vaccines of the present invention may also be administered via the oral route. In such cases the pharmaceutically acceptible excipient may also include alkaline buffers, or enteric capsules or microgranules. The vaccines of the present invention may also be administered by the vaginal route. In such cases, the pharmaceutically acceptable excipients may also include emulsifiers, polymers such as CARBOPOL®, and other known stablilisers of vaginal creams and suppositories. The vaccines of the present invention may also be administered by the rectal route. In such cases the excipients may also include waxes and polymers known in the art for forming rectal suppositories.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease or cancer, or allergy, or auto-immune disease. In a further aspect of the present invention there is provided an adjuvant combination and a vaccine as herein described for use in medicine. Vaccine preparation is generally described in New Trends and

Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

One embodiment of the present invention relates to the use of non-ionic surfactants such as a polyoxyethylene ether of general formula (I), and a polyoxyethylene sorbitan ester, in the manufacture of an adjuvant formulation. The present invention also relates to the use of a polyoxyethylene ether of general formula (I), and a polyoxyethylene sorbitan ester, and an antigen, in the manufacture of vaccine formulations. Optionally said adjuvant and vaccines manufactured as described, may further comprise an Octoxynol.

In an alternative related embodiment of the present invention the adjuvants of the present invention may further be combined with other adjuvants including Cholera toxin and its B subunit, Monophosphoryl Lipid A and its non-toxic derivative 3-de-O-acylated monophosphoryl lipid A (as described in UK patent no. GB 2,220,211), immunologically active saponin fractions *e.g.* Quil A derived from the bark of the South American tree Quillaja Saponaria Molina and derivatives thereof (for example QS21, US Patent No.5,057,540), and the oligonucleotide adjuvant system CpG (as described in WO 96/02555), especially $^{5'}$TCG TCG TTT TGT CGT TTT GTC GTT$^{3'}$ (SEQ ID NO. 1).

The present invention is illustrated by, but not limited to, the following examples.

Example 1, *Methods used to measure antibody (Ab) responses in sera*

ELISA for the measurement of influenza-specific serum Ig Abs in monkeys:

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 µl/well of 1 µg/ml HA of β-propiolactone (BPL) inactivated influenza virus (supplied by SSD GmBH manufacturer, Dresden, Germany) diluted in PBS. Free sites on the plates are blocked (1 hour, 37°C) using saturation buffer : PBS containing 1%BSA, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20). Then, serial 2-fold dilutions (in saturation buffer, 50 µl per well) of a reference serum added as a standard curve

(serum having a mid-point titer expressed as ELISA Unit/ml, and put in row A) and serum samples (starting at a 1/100 dilution and put in rows B to H) are incubated for 1hr 30mins at 37°C. The plates are then washed (×3) with washing buffer (PBS, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20)). Then, biotinylated goat anti-human Ig (Amersham) diluted 1/3000 in saturation buffer are incubated (50 µl/well) for 1hr 30mins, at 37°C. After 3 washings, and subsequent addition of streptavidin-horseradish peroxidase conjugate (Amersham), plates are washed 5 times and ~~incubated for 20 min at room temperature with 50 µl/well of revelation buffer (OPDA~~ 0.4 mg/ml (Sigma) and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). Revelation is stopped by adding 50 µl/well H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. Antibody titre are calculated by the 4 parameter mathematical method using SoftMaxPro software.

Hemagglutination Inhibition (HAI) activity of Flu-specific serum Abs in monkeys

In order to eliminate the non-specific inhibitors of hemagglutination present in the primate sera, these (25 µl) are incubated overnight at 37°C with 100 µl calcium chlorure/borate/sodium borate mix solution containing 400 receptor destroying enzyme units per ml of *V. cholerae* neuraminidase (Boerhinger Mannheim). After addition of 75 µl sodium citrate 2.5%, sera are heated for 30 minutes at 56°C. A solution of 50 µl PBS is added to give a final serum dilution of $1/10^{th}$. Then, 25 µl treated-sera are diluted in 25 µl PBS (serial 2-fold dilutions starting at 1/10) in 96 well Greiner plates. BPL inactivated whole virus is added (25 µl / well) at a concentration of 4 Hemagglutination Units (i.e. at a dilution which is 4-fold lower than the last one provoking an agglutination of red blood cells) for 30 minutes at room temperature (RT) under agitation. Chicken red blood cells are then added (25 µl / well) for 1 hour at RT. Plates are finally kept overnight at 4°C before to be read. The HAI titer corresponds to the inverse of the last serum dilution inhibiting the virus-induced hemagglutination.

Example 2, *Effect of Laureth 9 together with a combination of TWEEN80 and TritonX100 on the immunogenicity of an intranasal influenza vaccine in primed Rhesus monkeys*

The priming was done in Rhesus monkeys by administering with a spray device (under anesthesia) in each nostril 25 µg HA per strain of β-propiolactone-inactivated A/Beijing/262/95 and B/Harbin/7/94 influenza virus contained in 100 µl PBS. After ~~28 days, monkeys (4 or 5 animals/group) were boosted intranasally (under anesthesia)~~ with 200 µl of solution (100 µl per nostril, delivered with a spray device) containing 30 µg HA/strain of BPL-inactivated A/Beijing/262/95 and B/Harbin/7/94 influenza virus in either A: polyoxyethylene-9-lauryl ether 0.5% (L9); B: polyoxyethylene-9-lauryl ether 0.5% + TWEEN80 (0.11%) + triton-X-100 (0.074%); or by C: intramuscular injection of 15 µg HA/strain of an influenza vaccine containing the same strains as in A and B. Viral antigens were grown in eggs from seed stocks by the supplier (SSD GmBH, Dresden, Germany). HAI and Ig Ab responses were measured in sera as described in example 1. Results are expressed as percentages of animals having experienced a 4-fold Ab rise upon boosting.

Previous experience with 0.5% polyoxyethylene-9-lauryl ether has demonstrated that this formulation is potent in the induction of anti-influenza systemic immune responses. However, as shown in the table 1, this level of adjuvanticity is significantly improved by the addition of additional non-ionic surfactants. Thus when polyoxyethylene-9-lauryl ether is supplemented with TWEEN80 and triton-X-100, this formulation is capable of boosting pre-established systemic Ig Ab responses as efficiently as the classical parenteral influenza vaccine.

The haemagluttination inhibition (HAI) response is was also measured (table 2), once again, the best intranasal formulation is Polyoxyethylene-9-lauryl ether supplemented with TWEEN80 and triton-X-100. This formulation was equally as immunogenic as the conventional parenteral vaccine.

Table 1, serum Ig responses in monkeys

Ig ELISA antibodies	4-fold Ab rise seroconversion (%) to:	
Group	A/Beijing/262/95	B/Harbin/7/94
A	0	0
B	100	100
C	75	75

Table 2, serum HAI titers in monkeys

HAI antibodies	4-fold Ab rise seroconversion (%) to:	
Group	A/Beijing/262/95	B/Harbin/7/94
A	0	0
B	20	0
C	25	0

Claims

1. An adjuvant composition comprising (a) polyoxyethylene ether or ester of general formula (I):

$HO(CH_2CH_2O)_n$-A-R

wherein, n is 1-50, A is a bond or -C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl; and (b) at least one additional non-ionic surfactant.

2. An adjuvant composition as claimed in claim 1, wherein said additional non-ionic surfactant is a polyoxyethylene sorbitan ester or an Octoxynol.

3. An adjuvant composition as claimed in claim 1 wherein said additional non-ionic surfactant is a combination of a polyoxyethylene sorbitan ester and an Octoxynol.

3. An adjuvant composition as claimed in claim 2 or 3, wherein said polyoxyethylene sorbitan ester is polyoxyethylene sorbitan monooleate (TWEEN80™).

4. An adjuvant composition as claimed in claim 2 or 3, wherein said Octoxynol is t-octylphenoxypolyethoxyethanol (TRITON X100™).

5. A vaccine comprising an adjuvant as claimed in any one of claims 1 to 4, further comprising an antigen.

6. A vaccine as claimed in claim 5, wherein said antigen is selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Streptococcus, Mycoplasma, Mycobacteria, Haemophilus, Plasmodium or Toxoplasma, stanworth decapeptide; or Tumour associated antigens (TMA), MAGE, BAGE, GAGE, MUC-1, Her-2 neu, LnRH, CEA, PSA, KSA, or PRAME.

7. A vaccine as claimed in claim 6, wherein said antigen in an antigen or antigenic preparation from Influenza virus.

8. A vaccine as claimed in any one of claims 5 to 7, for use in medicine.

9. A method of producing a vaccine as claimed in claim 5 comprising admixing (a) polyoxyethylene ether or ester of general formula (I):

$HO(CH_2CH_2O)_n$-A-R

wherein, n is 1-50, A is a bond or -C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl;

(b) at least one additional non-ionic surfactant; and (c) an antigen.

10. Use (a) a polyoxyethylene ether or ester of general formula (I):

$HO(CH_2CH_2O)_n$-A-R

wherein, n is 1-50, A is a bond or -C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl;

(b) at least one additional non-ionic surfactant; and (c) an antigen;

in the manufacture of a medicament for the prophylaxis or treatment of disease.